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(21) (A1) 2,103,616
(22) 1993/08/09
(43) 1994/02/11

5,070,6/03

(51) INTL.CL.[5] C12N-015/55; C12N-009/78; C12N-015/74; C12P-007/44; C12N-001/21

(19)(CA) APPLICATION FOR CANADIAN PATENT (12)

(54) Polypeptides Possessing a Nitrilase Activity, DNA Sequence Coding for Said Polypeptides, Expression Cassettes and Host Microorganisms Enabling Them to Be Obtained, and Method of Converting Nitriles to Carboxylates by Means of Said Polypeptides

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CCA 3254 (10-92) 41 7530-21-936-3254

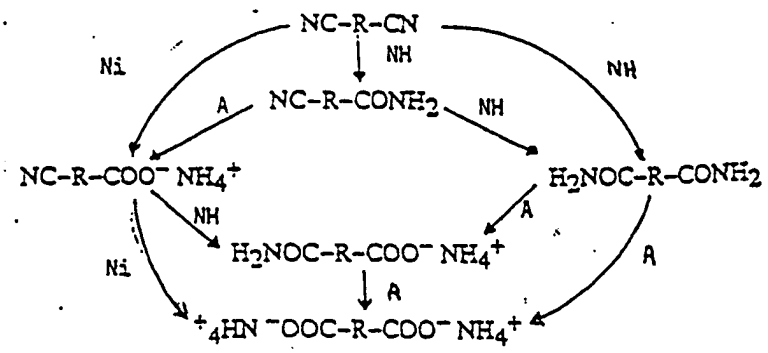
POLYPEPTIDES POSSESSING A NITRILASE ACTIVITY, DNA SEQUENCE CODING FOR SAID POLYPEPTIDES, EXPRESSION CASSETTES AND HOST MICROORGANISMS ENABLING THEM TO BE OBTAINED, AND METHOD OF CONVERTING NITRILES TO CARBOXYLATES BY MEANS OF SAID POLYPEPTIDES

The present invention relates to novel polypeptides having a nitrilase activity and to the genetic engineering tools for producing them, namely a DNA sequence, the expression cassettes carrying this recombinant DNA sequence, and the recombinant microorganisms (host microorganisms) containing said DNA sequence.

The present invention further relates to an enzymatic method of converting nitriles to carboxylates by means of the polypeptides according to the invention or a host microorganism containing the DNA sequence according to the invention. One particular application of the method of the invention is the enzymatic synthesis of ammonium adipate or ammonium 5-cyanovalerate by the hydrolysis of adiponitrile with the aid of a polypeptide or host microorganism according to the invention.

Ammonium adipate is known to be a particularly valuable product because it can be converted to adipic acid, a product which is itself widely used for the preparation of nylon 6,6.

The enzymatic hydrolysis of dinitriles has been described by numerous authors. However, the routes by which these dinitriles are hydrolyzed to organic acids are not often referred to. The theoretical hydrolysis scheme is as follows:



NH = nitrile hydratase

Ni = nitrilase

A = amidase

R = $(CH_2)_n$, n being an integer equal to 4 in the case of adiponitrile.

In actual fact, it is very often observed that certain routes are preferred and that certain products are not formed or else are not hydrolyzed.

Among the microorganisms for which it has been possible to demonstrate the existence of an enzymatic activity permitting this hydrolysis, there may be mentioned in particular the strains belonging to the genus *Fusarium*, which degrade succinonitrile and adiponitrile, although the reaction products are not indicated [Goldlust *et al.*, Biotechnol. and Appl. Biochem., 1989, 11, 581]; the strains belonging to the genus *Pseudomonas*, which degrade adiponitrile [Yanase *et al.*, Agric. Biol. Chem., 1982, 46, 2925]; and the strains belonging to the genus *Rhodococcus*, in particular *Rhodococcus rhodochrous* NCIB 11 216, which hydrolyzes adiponitrile to adipic acid [Bengis-Garber *et al.*, Appl. Microbiol. Biotechnol., 1989, 32, II], and also *Rhodococcus rhodochrous* K22, whose nitrilase permits the hydrolysis of adiponitrile and glutaronitrile

[Yamada *et al.*, J. Bacteriol., 1990, 172 (9), 4807-4815], albeit with a low activity ratio compared with that for the hydrolysis of aromatic nitriles.

Consequently, it can be seen that the enzymatic hydrolysis of dinitriles is rather complex: in all cases, although the first CN group is hydrolyzed by the enzyme, the second group is not hydrolyzed at all in some cases, or else is hydrolyzed at a very low rate in other cases.

It has now been found that it is possible to hydrolyze nitriles to carboxylates, and more particularly dinitriles to carboxylates or dicarboxylates, totally and rapidly, by using appropriately selected enzymes either as such or, preferably, in the form of recombinant microorganisms which generate them.

The present invention therefore relates to novel polypeptides having a nitrilase activity which have been isolated from a strain of *Comamonas testosteroni*. More precisely, these polypeptides are prepared by extraction and purification from cultures of natural or recombinant microorganisms, the purification being effected by a series of steps consisting in preparing an enzymatic extract from the cell culture, precipitating this extract with ammonium sulfate and purifying it by different steps involving chromatography and gel filtration. These steps, which employ techniques well known to those skilled in the art, are described in detail in the illustrative Examples below.

In the present description, "nitrilase activity" denotes the direct conversion of a nitrile to an ammonium carboxylate, the corresponding amide not being a substrate for the enzyme.

The invention further relates to a DNA sequence coding for a polypeptide having a nitrilase activity. The DNA sequence coding for a polypeptide of the

invention can be selected from:

- the DNA sequence coding for a polypeptide having a nitrilase activity, as shown in Fig. 4,
- an analog of this sequence resulting from the degeneracy of the genetic code,
- or else a DNA sequence hybridizing with one of these sequences or with a fragment thereof and coding for a polypeptide having a nitrilase activity.

Such a DNA sequence can be obtained by cloning the genomic DNA fragment coding for the desired polypeptide, with the aid of nucleotide probes produced from the purified polypeptide.

The invention further relates to the expression cassettes which carry the above-defined recombinant DNA sequence together with the signals ensuring its expression. These expression cassettes can either be integrated in the genome of the host or located on an expression vector such as a plasmid containing a selection means.

In particular, these expression cassettes contain transcription and translation initiation regions which contain a ribosome binding site and a promoter sequence. These regions may be homologous or heterologous with the microorganism which naturally produces the polypeptide.

The choice of these regions depends especially on the host used. In particular, when the host microorganisms are procaryotic, the heterologous promoter can be selected from strong bacterial promoters such as the tryptophan operon promoter Ptrp of *E. coli*, the lactose operon promoter Plac of *E. coli*, the phage lambda right promoter P_R, the phage lambda left promoter P_L, the strong promoters of *Pseudomonas* and *Comamonas* and the strong promoters of *Corynebacteria*.

More particularly, in the case of the phage

lambda right promoter, the thermosensitive form P_RCIts may be preferred. In the case of eucaryotic micro-organisms such as yeasts, the promoters can originate from glycolytic yeast genes such as the genes coding for phosphoglycerate kinase (PGK), glyceraldehyde-3-phosphate dehydrogenase (GPD), lactase (LAC4) and enolase (ENO).

As far as the ribosome binding sites are concerned, the one derived from the lambda CII gene, as well as those derived from homologous genes of *Comamonas* or *Pseudomonas* or those derived from genes of *Corynebacteria*, are used preferentially when the host microorganism is procaryotic.

A region permitting a termination of the translation and functional transcription of the envisaged host can be positioned at the 3' end of the coding sequence. The expression cassette also comprises one or more markers making it possible to select the recombinant host. The preferred markers are dominant markers, *i.e.* those conferring a resistance to antibiotics such as ampicillin or streptomycin, or to other toxic products.

Enterobacteria such as *E. coli*, bacteria belonging to the genera *Comamonas* or *Pseudomonas*, and corynebacteria such as those belonging to the genera *Corynebacterium*, *Brevibacterium* or *Rhodococcus*, may be mentioned in particular among the host microorganisms used.

The invention further relates to the micro-organisms containing the recombinant DNA sequence according to the invention, for example on a plasmid containing a selection means.

A recombinant microorganism containing said DNA sequence on a plasmid structure was deposited in the Collection Nationale de Cultures de Micro-organismes

(C.N.C.M.) (Institut Pasteur, 25 rue du Docteur Roux, Paris) under no. I-1242 on 21st July 1992. This microorganism is the strain *E. coli* TG1, which contains plasmid pXL2148; this microorganism is also identified by the Applicant using the reference G4207.

The invention further relates to the microorganisms capable of converting nitriles to carboxylates, and more particularly aliphatic dinitriles of the formula NC-R-CN, in which R is a linear or branched alkylene group having from 1 to 10 carbon atoms, to carboxylates.

Beyond acquiring their structures I and II during their synthesis by the microorganisms according to the invention, it is important that the polypeptides in question stabilize in their structures III and IV so as to possess an optimal nitrilase activity.

The Applicant takes credit for having discovered means for favoring the above-mentioned stabilization.

Thus any microorganism according to the invention preferably contains:

- at least one protein agent for assisting the folding of the polypeptides which the microorganism synthesizes, and in particular the nitrilase referred to in the present disclosure,
- and/or the genes coding for such an agent, this agent being present in a greater amount than that corresponding to the base level of the microorganism in question.

In terms of the present invention, base level is understood as meaning the maximum level which can be attained by the corresponding wild-type microorganism in question.

Advantageously, this agent is the GroE chaperone of *E. coli* or its homolog of eucaryotic or pro-

caryotic origin.

The GroE chaperone of *E. coli* is normally present in the wild-type strains.

The genes coding for the agent are carried by the chromosome or by an extrachromosomal element (plasmid, phage). They are preferably amplified by any known and appropriate means so as to favor the synthesis of the agent in the microorganism.

The genes coding for the agent are under the dependence of expression systems homologous or heterologous with their host microorganism.

The invention further relates to the method of converting nitriles to carboxylates with the aid of a polypeptide according to the invention or a recombinant microorganism which generates it. This method consists in bringing the nitrile to be converted into contact with a polypeptide or recombinant microorganism as defined above. The process is generally carried out at room temperature. In one particular embodiment of the invention, the polypeptide or recombinant microorganism is immobilized on or in a solid support.

The method of the invention is suitable for the conversion of nitriles to carboxylates and more particularly for the conversion of dinitriles of the formula NC-R-CN, in which R is a linear or branched alkylene group containing 1 to 10 carbon atoms, to carboxylates.

The method of the invention is particularly appropriate for the enzymatic synthesis of ammonium adipate from adiponitrile.

The Examples which follow afford an illustration of the characteristics and advantages of the present invention without however limiting its scope.

DESCRIPTION OF THE FIGURES

Fig. 1 shows the yield (%) of the hydrolysis of adiponitrile to cyanovalerate (curve a) and to ammonium adipate (curve b) as a function of the reaction time in hours for the strain *Comamonas testosteroni* sp.

Fig. 2a and 2b show the restriction maps of plasmids pXL2075 and pXL2076.

Fig. 3 shows the restriction map of the SstI-SstI fragment of 4.1 kb containing the DNA sequence (called "nitrilase gene" in the Figure) coding for the polypeptide having the nitrilase activity according to the invention, said fragment being present in plasmids pXL2075 and pXL2076. The strategy for producing the XbaI-SstI fragment containing the DNA sequence according to the invention is also shown in this Figure.

Fig. 4 shows the DNA sequence according to the invention with its deduced amino acid sequence.

Fig. 5 shows the restriction map of plasmid pXL2087.

Fig. 6 shows the restriction map of plasmid pXL2148.

Fig. 7 shows the SDS-PAGE, 10% SDS, indicating the expression of the DNA sequence according to the invention in the strain *E. coli* TG1/pXL2027. Each lane corresponds to an amount of protein equivalent to 60 µl of culture at an optical density of 3 at 610 nm.

Fig. 8 shows the restriction map of plasmid pXL2158.

Fig. 9 shows the SDS-PAGE, 12.5% SDS, indicating the expression of the DNA sequence according to the invention in the strains TG1/pXL2158 and TG1/pXL2158 + pXL2035 (GroE).

Fig. 10 shows the restriction map of plasmid pXL2169.

Fig. 11 shows the SDS-PAGE, 10% SDS, indicating the expression of the DNA sequence according to the invention in the strain *Pseudomonas putida* G2081-pXL2169.

The abbreviations used in the remainder of the description have the following meanings:

SSC: buffer commonly used for hybridizations, containing sodium citrate and NaCl (20x SSC = 3 M NaCl, 0.3 M sodium citrate, pH 7)
SDS: sodium dodecylsulfate
FPLC: fast protein liquid chromatography
SDS-PAGE: sodium dodecylsulfate/polyacrylamide gel electrophoresis
IPTG: isopropyl β-D-thiogalactopyranoside

EXAMPLES

EXAMPLE 1: PURIFICATION OF THE NITRILASE OF *Comamonas testosteroni* sp.

1 - PREPARATION OF THE CELLS:

A strain of *Comamonas testosteroni* sp. was cultivated in a shake flask, at 28°C, for 15 h 30 min, in medium A having the following composition:

Medium A

- Glucose	5	g/l
- $(NH_4)_2SO_4$	1	g/l
- Na_2HPO_4	5.24	g/l
- $KHPO_4$	2.77	g/l
- Yeast extract	5	g/l
- Casamino acids	1	g/l

This preculture was used to inoculate a 20 l fermenter containing 15 l of medium A. The pH, temperature, air flow rate and shaking speed were set to 6.6, 28°C, 300 l/h and 350 rpm respectively. After

24 h, 84 g of wet cells were harvested. This corresponds to a content by dry weight of cells of 0.9 g/l and to an optical density at 660 nm (OD_{660nm}) of 2.

2 - DETERMINATION OF THE ENZYMATIC ACTIVITY ON ADIPONITRILE:

A cellular residue containing 13.1 mg of dry weight of cells was suspended in 2 ml of a 52.3 mM solution of adiponitrile in 50 mM potassium phosphate buffer, pH 7. The reaction was carried out at 25°C, with shaking, and the kinetics were followed by sampling. 5-Cyanovaleramide, adipamide, 5-cyanovalerate, adipamate and adipate were determined on each sample by high performance liquid chromatography (HPLC). The results are collated in Fig. 1, which shows the curves of the yield (on the ordinate) of cyanovalerate (curve a) and ammonium adipate (curve b). The respective rates of formation of cyanovalerate and adipate were greater than 0.45 and equal to 0.15 U/mg of dry weight of cells (1 U is equal to 1 µmol of product formed per minute).

3 - PURIFICATION:

All the purification steps were carried out in 50 mM Tris/HCl buffer, pH 7.5, 1 mM dithioerythritol (DTE), unless indicated otherwise. At each step, the nitrilase activity of the fractions was determined at pH 7 and at 25°C in 10 mM phosphate buffer in the presence of 10 mM adiponitrile. The protein concentration of the pools was determined by the Coomassie blue method (PIERCE Protein assay kit). The proteins were analyzed by SDS-PAGE (Phastsystem, PHARMACIA).

The procedures of each step are discussed below.

Step 1: Crude extract

57 g of wet cells were taken up in 85 ml of buffer and treated with ultrasound for 30 min (VIBRA-CELL sonicator from Bioblock: probe 13 mm; power 7; 40% of the cycle active). The OD_{600nm} thus dropped from 97 to 60. After centrifugation at a maximum of 48,000 g for 60 min, the supernatant was recovered.

This supernatant was brought to 15% saturation by the gradual addition of ammonium sulfate. After 1 h, the suspension was centrifuged for 30 min at a maximum of 30,000 g. The supernatant was brought to 50% saturation. After 1 h, the suspension was centrifuged under the same conditions and the precipitate was recovered and then dialyzed against the buffer for two days.

Step 2: Ion exchange column (Q Sepharose Fast Flow)

The dialyzed fraction was loaded at a rate of 125 ml/h on to a column (26 x 380 mm) of "Q Sepharose Fast Flow" equilibrated with the buffer at a rate of 250 ml/h. The column was percolated at a rate of 250 ml/h by the following solutions in succession:

- 166 ml of buffer
- 180 ml of a gradient of 0 to 0.2 M KCl in the buffer
- 180 ml of buffer to which 0.2 M KCl had been added
- 270 ml of a gradient of 0.2 to 0.4 M KCl in the buffer
- 180 ml of buffer to which 0.4 M KCl had been added
- 200 ml of buffer to which 1 M KCl had been added

The fraction having the nitrilase activity was eluted in a volume of 129 ml during the 0.2 M KCl stage.

The following steps are carried out on the FPLC system (Pharmacia).

Step 3: Gel filtration (FPLC Superdex 200)

The previously obtained fraction having the nitrilase activity (129 ml) was concentrated to 12 ml by precipitation of the proteins with ammonium sulfate at 80% saturation, followed by dialysis against the buffer. The fraction concentrated in this way (12 ml) was loaded in 2 batches on to the column of gel (16 x 600 mm) equilibrated with the buffer to which 0.1 M KCl had been added, at a rate of 0.8 ml/min. The fractions having the nitrilase activity were eluted with the above buffer at a rate of 1 ml/min and in a total volume of 36 ml. These fractions correspond to a molecular weight of 280 kDa.

Step 4: Column of hydroxyapatite (BIO-RAD HPHT; 7.8 x 100 mm)

The fractions obtained above were concentrated to 8 ml by ultrafiltration (DIAFLO PM39 membrane, AMICON). The concentrated solution was injected on to the column of hydroxyapatite equilibrated with the buffer to which 10 μM $CaCl_2$ had been added. The column was percolated at a rate of 0.5 ml/min with the following in succession:

- 5 ml of equilibration buffer
- 15 ml of a gradient of 0 to 350 mM potassium phosphate in the equilibration buffer
- 10 ml of the equilibration buffer to which 350 mM potassium phosphate had been added

The fractions having the nitrilase activity were eluted between 62 and 135 mM potassium phosphate in a volume of 3 ml.

Step 5: Hydrophobic interaction column (FPLC-Phenyl Superose HR 5/5)

The active fractions obtained above, brought to 15% saturation with ammonium sulfate, were loaded at a rate of 0.5 ml/min on to the column equilibrated with

buffer containing ammonium sulfate at 15% saturation. The column was percolated with:

- 6 ml of equilibration buffer
- 12 ml of a decreasing ammonium sulfate gradient of 15% to 0% saturation in the buffer
- 23 ml of buffer

Some of the fractions having the nitrilase activity were eluted during the washing of the column with the equilibration buffer. These active fractions were reinjected under the same conditions. This operation was performed twice. The active fractions eluted after the gradient were pooled (volume 51 ml).

Step 6: Gel filtration (FPLC-Superdex 200)

The 51 ml were concentrated to 3 ml by ultrafiltration on a membrane (DIAFLO PM30, AMICON). These 3 ml were loaded on to the column (16 x 600 mm) equilibrated with the buffer to which 0.1 M KCl had been added. The 9 ml containing the activity were eluted at a position corresponding to a molecular weight of 280 kDa. This solution was brought to 36% with glycerol and then frozen for 15 days.

Step 7: Ion exchange column (FPLC Mono Q HR 5/5)

The protein solution was thawed and loaded on to the column equilibrated with the buffer containing 0.1 M KCl, at a rate of 0.5 ml/min. The column was percolated with the following in succession:

- 15 ml at 0.5 ml/min of buffer to which 0.1 M KCl had been added
- 4.5 ml at 1 ml/min of buffer to which 0.1 M KCl had been added
- 15 ml at 1 ml/min of a gradient of 0.1 to 0.4 M KCl in the buffer
- 10 ml of buffer to which 0.4 M KCl had been added

The active fractions were eluted between 0.15

and 0.3 M KCl. These fractions are homogeneous. SDS-PAGE analysis reveals two bands very close to 38 and 39 kDa. The fractions thus obtained will hereafter be called "purified nitrilase".

The data from each of the above purification steps are collated in Table 1 below:

TABLE 1: PURIFICATION OF THE NITRILASE OF *Comamonas testosteroni* sp.

PURIFICATION STEP	Vol. ml	Protein mg	ACTIVITY		YIELD		PF
			Total	Specific	Protein	Activity	
0 - Crude extract	61	920	62,000	68	100	100	1
2 - Q Sepharose FF	130	245	47,000	190	27	76	2.8
3 - Gel filtration	36	27	56,000	2100	2.9	90	30
4 - Hydroxyapatite column	3	12	49,000	4100	1.3	79	60
5 - Phenyl Superose	51	11	11,000	1000	1.1	18	15
6 - Gel filtration	9	2.7	6,300	2300	0.3	10	34
7 - Mono Q HR 5/5	2.9	1	1,200	1200	0.01	2	18

ABBREVIATIONS: PF = purification factor; U = 1 µmol/h

4 - DETERMINATION OF THE N-TERMINAL SEQUENCE OF THE NITRILASE:

Taking the purified protein, the N-terminal sequence of 27 amino acids was determined by Edman automatic sequential degradation using an "Applied Biosystems Model 470 A" apparatus. This sequence is as follows:

Met Lys Asn Tyr Pro Thr Val Lys Val Ala Ala Val Gln Ala Ala Val
5 10 15
Phe Met Asn Leu Glu Ala Thr Val Asp Lys Thr
20 25

A search of sequence libraries made it possible to find a 53% identity with the nitrilase of *Klebsiella pneumoniae* active on bromoxynil, which forms the subject of European patent application no. 373 173.

5 - ACTIVITY OF THE PURIFIED NITRILASE:

a) - Influence of the pH on the activity of the nitrilase:

The purified nitrilase was tested at different pH values on two substrates, adiponitrile and 5-cyanovalerate, under the conditions indicated in Table 2 below.

TABLE 2: ACTIVITY OF THE PURIFIED NITRILASE ON ADIPONITRILE AND CYANOVALERATE AS A FUNCTION OF THE pH

SUBSTRATE	BUFFER		SPECIFIC ACTIVITY U/mg of protein
	Nature	pH	
Adiponitrile	Acetate	3.0	2300
	Acetate	4.0	2900
	Acetate	4.5	2800
	Acetate	5.0	2700
	Phosphate	6.0	2900
	Phosphate	7.0	2700
	Phosphate	8.0	2800
5-Cyanovalerate	Acetate	4.0	450
	Acetate	5.5	180
	Phosphate	7.0	30
	Phosphate	8.0	6

COMMON CONDITIONS: [substrate] = 10 mM; buffer 10 mM; T 25°C;

[nitrilase] = 12 µg/ml for cyanovalerate and 3 µg/ml for adiponitrile (fraction, step 6); U (adiponitrile) = µmol of cyanovalerate formed/h, U (cyanovalerate) = µmol of adipate formed/h

b) - Activity range of the purified nitrilase:

The activities of the purified nitrilase were measured on adiponitrile, 5-cyanovaleramide, 5-cyanovaleric acid, benzonitrile, propionitrile and acrylonitrile. The results are given in Table 3.

TABLE 3: RELATIVE ACTIVITY OF THE PURIFIED NITRILASE ON VARIOUS NITRILES

SUBSTRATE	RELATIVE ACTIVITY (%)
Adiponitrile	100
5-Cyanovaleramide	28
5-Cyanovaleric acid	22
Acrylonitrile	23
Propionitrile	6
Benzonitrile	4

COMMON CONDITIONS: acetate buffer 10 mM, pH 4; substrate 10 mM; volume = 3 ml; T = 25°C; reaction time = 1 or 3 h; proteins: from 5 to 30 µg/ml

EXAMPLE 2: CLONING OF THE NITRILASE OF *Comamonas testosteroni* sp.

A nucleotide probe was synthesized from the NH_2-terminal sequence presented in Example 1; the high percentage of GC in the strains of *Comamonas* described in the literature (Tamaoka *et al.*, Int. J. Syst. Bacteriol., 1987, 37, 52-59) dictated a choice for the third position of the codon in the case of lysines and

in the case of valine. The probe is a 26 mer of degeneracy 128 (N replaces A, C, G or T):

```
      M  K  N  Y   P  T  V  K   V
   5' ATGAAGAATT ATCCNACNGT CAAGGT 3'
                 C  C        G
```

The strategy followed consisted first of all in verifying the specificity of this nucleotide probe and determining the nature of the genomic DNA fragments to be cloned. Briefly, the genomic DNA of *Comamonas testosteroni* sp. was digested with several restriction enzymes (SstI, SphI, BamHI, PstI *etc.*) corresponding to sites usable for cloning.

After electrophoresis on agarose gel and transfer to a nylon membrane, the various digestions were hybridized with the probe. The probe is found to have a sufficient specificity under the hybridization conditions used (hybridization buffer = 5x SSC, 5x Denhardt, 0.1% SDS, 50 mM Na_3PO_4, pH 6.5, 250 µg/ml of ssDNA; hybridization temperature 50°C; washing conditions: 1 h, 6x SSC, room temperature, and 5 min, 2x SSC, 0.1% SDS, 50°C).

Under these conditions, the probe made it possible to obtain important signals without ambiguity, in particular in the case of digestions with SstI, SphI, BamHI and PstI. The hybridization blots show in particular the existence of a single SstI-SstI fragment of about 4 kb. To clone this fragment, the fragments of 3.5 to 4.5 kb from an SstI digestion of the genomic DNA were purified by preparative electrophoresis on agarose and electroelution and then ligated to plasmid pUC19 (YANISCH *et al.*, Gene, 33 (1985) 103), itself digested with SstI. After transformation in the strain DH5α (Clontech Laboratory, Palo Alto, California), 600 white

clones on LB amp X-gal (SAMBROOK *et al.*, Molecular Cloning, A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor Laboratory, N.Y., 1989) were subcultured individually, transferred to a nylon membrane and then analyzed by hybridization with the probe used to hybridize the Southern blot, under the same conditions of stringency. Six clones were thus identified as hybridizing very strongly with the probe. Two clones which had inserted the same fragment of about 4.1 kb in both orientations (pXL2075 [Fig. 2a] and pXL2076 [Fig. 2b]) were analyzed in greater detail (restriction mapping, partial sequencing using the probe as primer, and Southern blot). It was thus possible to show that the 5' part of the gene which hybridizes with the probe is located on an XhoI-XbaI fragment of about 150 bp orientated in the XhoI to XbaI direction. Fig. 2a and 2b show the restriction maps of these plasmids.

EXAMPLE 3: SEQUENCE OF A FRAGMENT OF 1194 bp CONTAINING THE DNA CODING FOR THE POLYPEPTIDE HAVING THE NITRILASE ACTIVITY

The location, on the cloned insert, of the fragment of 1194 bp containing the sequenced nitrilase gene is indicated in Fig. 3. The strategy for the sequencing of this fragment, performed by conventional methods known to those skilled in the art, is also indicated in Fig. 3. The various sequences were all obtained by the chain termination method (sequenase kit in the presence of 7-deaza-dGTP; (^{35}S)dATP either on single-stranded matrices of recombinant M13 (mp 18 or 19, see YANISCH *et al.*, *op. cit.*) carrying subfragments, or directly on plasmid pXL2075). Several specific primers were also synthesized for this purpose.

The DNA sequence according to the invention is shown in Fig. 4. The average G+C content of the sequence obtained is 45.7%, which is lower than the G+C content of 61.5% described for other strains of *Comamonas* (Tamaoka *et al.*, *op. cit.*). An analysis of the sequence obtained made it possible to characterize an open reading frame of 1064 bp, hereafter called the nit gene, coding for a polypeptide of 354 residues corresponding to a molecular weight of 38,725 Da. The amino acid sequence of this polypeptide is indicated in Fig. 4. This polypeptide comprises the NH_2-terminal sequence used to synthesize the probe, as well as three internal sequences determined on tryptic fragments of the purified nitrilase (these internal sequences are underlined in Fig. 4).

This open reading frame thus represents the DNA sequence according to the invention.

EXAMPLE 4: HOMOLOGY WITH OTHER PROTEINS, IDENTIFICATION OF HOMOLOGOUS SEQUENCE

The DNA sequence according to the invention was compared with all the sequences in the NBRF protein library; only one significant homology was found with the nitrilase of *Klebsiella ozaenae* specific for the herbicide bromoxynil (Stalker *et al.*, J. Biol. Chem., 1988, 263, 6310-6314). The two nitrilases exhibit a strict homology of 34.9% distributed over 320 amino acids. Furthermore, this protein exhibits a strict homology of 34.4%, distributed over 312 amino acids, with the nitrilase of *Arabidopsis* specific for indole-3-acetonitrile [Bartling *et al.*, Eur. J. Biochem., 205, 417-424, 1992].

EXAMPLE 5: EXPRESSION OF THE NITRILASE IN *E. coli*

To confirm the identification of the coding frame with the purified nitrilase, the nit gene, preceded by its own ribosome binding site, was placed under the control of the lactose operon promoter of *E. coli* in accordance with the procedure described below: Plasmid pXL2087, described in Fig. 5, was obtained by insertion of the XhoI-NcoI fragment derived from plasmid pXL2075 between the corresponding sites of vector pMTL25 (Chambers *et al.*, Gene, 1988, 68, 139-149). This plasmid therefore contains the lactose operon promoter Plac, followed by the ribosome binding site and the structural nitrilase gene, as well as a gene conferring ampicillin resistance.

The expression of the nitrilase was visualized in the strain *E. coli* TG1 containing plasmid pXL2087. For this purpose, the strain TG1/pXL2087 and the control strain TG1/pUC19 were cultivated for 16 h at 37°C in LB medium (Miller, J.H., 1972, Experiments in Molecular Genetics - Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) containing 100 µg/ml of ampicillin, and then diluted 100-fold in the same medium and at the same temperature. When the cultures had reached an OD_{610} of between 0.5 and 1, IPTG was added at a final concentration of 1 mM. After 2 h of culture, the bacteria were collected.

After sonication of the cells, the expression of the nitrilase was measured by SDS-PAGE in the crude fraction or, after centrifugation, in the residue and the supernatant. The results are presented in Fig. 7 and show a high level of expression of the nitrilase in the extracts of cells cultivated in the presence of IPTG; however, this protein is essentially in insoluble form.

In Fig. 7, M represents the molecular weight marker; the molecular weights are indicated in kDa. Also, the lanes have the following meanings:

	TG1 + pUC19	TG1 + pUC19 + IPTG	TG1 + pXL2087	TG1 + pXL2087 + IPTG
Crude fractions	A	D	G	J
Residues	B	E	H	K
Supernatants	C	F	I	L

Starting from plasmid pXL2087, plasmid pXL2148 was prepared by insertion of the XhoI-EcoRI fragment of plasmid pXL2087, carrying the gene coding for the nitrilase, between the SalI and EcoRI sites of pBR322 [SUTCLIFFE, Nucleic Acid Res., 5 (1978) 2721-2730].

This plasmid pXL2148, whose restriction map is shown in Fig. 6, was also used to transform the strain *E. coli* TG1 by the calcium chloride method. The micro-organisms were selected on ampicillin. The strain *E. coli* TG1 (pXL2048) (G4207) transformed in this way was deposited in the Collection Nationale de Cultures de Micro-organismes in Paris (Institut Pasteur, 25 rue du Docteur Roux) under no. I-1242 on 21st July 1992. Other expression systems were used to produce the nitrilase in a recombinant microorganism.

First of all, the nit gene was expressed in *E. coli* behind the tryptophan operon promoter of *E. coli* under the dependence of the RBS of the phage λ CII gene. To do this, an NdeI restriction site was created on the initiation codon of nit, and the NdeI/AhaII fragment of 117 bp, containing the 5′ part of the nit gene, was amplified by the PCR technique starting from pXL2087. An NdeI/XbaI fragment of 61 bp, obtained

after digestion of the first fragment, was ligated to the EcoRI/NdeI fragment containing the tryptophan operon promoter of *E. coli* and the ribosome binding site of the bacteriophage λ CII gene (Ptrp-RBSCII) between the EcoRI and XbaI sites of pUC19 (Yanisch *et al.*, Gene, 33 (1985) 103) to give plasmid pXL2149. The EcoRI/XbaI fragment of pXL2149, containing the 5' part of nit behind Ptrp-RBSCII, was ligated to the XbaI/SalI fragment of pXL2087 containing the 3' part of the nit gene between the EcoRI and SalI sites of pXL642 (Mayaux, unpublished results): pXL642 is a derivative of pXL534 (Latta *et al.*, 1990, DNA Cell Biol., 9, 129) in which the superexpressed gene codes for a tissue inhibitor of metalloproteases and in which the HindIII site downstream from the superexpressed gene has been replaced by the EcoRI/HindIII multisite of M13mp18.

The final plasmid pXL2158 is therefore a derivative of pBR322 (Sutcliffe, Nucleic Acid Res., 5 (1978) 2721) containing a gene conferring ampicillin resistance and the nit gene under the control of Ptrp-RBSCII. The restriction map of this plasmid pXL2158 is shown in Fig. 8.

Plasmid pXL2158 was used to transform the strain *E. coli* TG1. The strain TG1/pXL2158 and the control strain TG1 containing vector pMTL22 were cultivated for 16 h at 30°C in M9 glucose medium (Miller, J.H., 1972, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) containing 100 µg/ml of ampicillin and 100 µg/ml of tryptophan. These cultures were diluted 100-fold in the same medium, but without tryptophan, and cultivated for 6 hours at the same temperature.

After sonication of the cells, the expression of the nitrilase of *Comamonas testosteroni* NI 1 was measured in 12.5% SDS/polyacrylamide gel in the crude

fraction or, after centrifugation, in the residue and the supernatant. The results are shown in Fig. 9.

	TG1/pXL2158 + pXL2035	TG1/pXL2158	TG1 + pMTL22
Supernatant	A	D	G
Residue	B	E	H
Total extract	C	F	I

This gel shows that pXL2158 induces a strong expression of the nitrilase, predominantly in insoluble form.

The efficacy of the GroE chaperone was then tested (Hemmingsen *et al.*, 1988, Nature, 333, 330) in order to assist the correct folding of the nitrilase. For this purpose, plasmid pXL2035 was constructed in the following manner: The EcoRI/HindIII fragment of 2.2 kb, containing the groES and groEL genes coding for the two subunits of GroE, was extracted from plasmid pOF39 (Fayet *et al.*, 1986, Mol. Gen. Genet., 202, 435) and introduced between the EcoRI and HindIII sites of vector pDSK519 (Keen *et al.*, 1988, Gene, 70, 191).

Plasmid pXL2035 was introduced into the strain TG1 containing pXL2158. The resulting strain was cultivated under the same conditions as before, in the presence of 50 mg/l of kanamycin; the expression results are visualized in Fig. 9. It is found that the superexpression of GroE (only the GroEL subunit is visible on the gel) solubilizes the bulk of the nitrilase expressed from pXL2158.

The same expression system was used to produce the nitrilase in *Pseudomonas putida*. Thus, starting from pXL2158, the NdeI/NcoI fragment of 1256 bp and the

NcoI/BamHI fragment of 535 bp were introduced between the NdeI and BamHI sites of pXL1841. pXL1841 (Blanche *et al.*, 1991, J. Bacteriol., 173, 4637) is a derivative of pKT230 (Bagdasarian *et al.*, 1981, Gene, 15, 237) expressing a *Methanobacterium ivanovii* gene behind Ptrp-RBSCII.

The final plasmid pXL2169 is therefore a derivative of pKT230 containing a gene conferring kanamycin resistance and the nit gene under the control of Ptrp-RBSCII (*cf.* Fig. 10). This plasmid was introduced into the strain *Pseudomonas putida* G2081. G2081 is a derivative of *Pseudomonas putida* KT2440 (Bagdasarian and Timmis, 1981, in Hofschneid and Goebel, Töpics in Microbiology and Immunology, 47, Springer Verlag, Berlin) rendered resistant to nalidixic acid and rifampicin. Vector pDSK519 (Keen *et al.*, 1988, Gene, 70, 191) was used as the control plasmid. G2081 (pXL2169) and the strain G2081 (pDSK519) were cultivated overnight at 30°C in LB medium containing 20 mg/l of kanamycin. These precultures were diluted 100-fold in the same medium. The cultures were then continued for 7 h 30 min at the same temperature. After sonication of the cells, the expression of the nitrilase of *Comamonas testosteroni* NI 1 was measured in 10% SDS/polyacrylamide gel in the crude fraction or, after centrifugation, in the residue and the supernatant. The results are presented in Fig. 11. Only the crude extract of the strain G2081 (pDSK519) was deposited (well D); for the strain G2081 (pXL2169), the total extract, the sonication residue and the sonication supernatant were deposited in wells C, B and A respectively. This experiment shows that the strain of *Pseudomonas putida* expresses large amounts of nitrilase in soluble form.

EXAMPLE 6: ASSAY OF THE NITRILASE ACTIVITY OF RECOMBINANT STRAINS

The Examples which follow illustrate the nitrilase activity of the recombinant strains *E. coli* TG1 and *Pseudomonas putida* G2081.

The different plasmids integrated into these strains are as follows:

PLASMID	CHARACTERISTICS
pXL2087	Recombinant plasmid which carries the *Comamonas* NI 1 nitrilase gene under the control of the promoter P_{lac}.
pXL2158	Recombinant plasmid which carries the *Comamonas* NI 1 nitrilase gene under the control of the tryptophan promoter.
pXL2035	Recombinant plasmid which carries the genes coding for GroE and S.
pXL2169	Broad host range plasmid with an insertion, carrying the *Comamonas* NI 1 nitrilase gene under the control of P_{trp}.
pDSK519	Control plasmid (*cf.* page 24 line 18).

The activities of these strains, induced or non-induced, are measured on adiponitrile and 5-cyanovalerate at different pH values and are compared with the negative control strains: *E. coli* TG1, *E. coli* TG1 (pXL2035) and *Pseudomonas putida* G2081.

1 - PREPARATION OF THE CELLS:

The cultures are carried out under the conditions described in Table 4. During the exponential growth phase, one of the two cultures of the recombinant strain is induced with 1 mM IPTG; after 2 h at 37°C, this culture is treated.

TABLE 4: CULTURE OF THE STRAINS

MICROORGANISM	MEDIUM	OD_{660nm}	DW (g/l)
1 - *E. coli* TG1	a	3.1	0.90
2 - *E. coli* (pXL2087)	b	3.2	0.90
3 - *E. coli* (pXL2087)	c	2.5	0.90
4 - *E. coli* (pXL2035)	d	2.1	0.90
5 - *E. coli* (pXL2148)(1)	b	3.1	0.80
6 - *E. coli* (pXL2035, 2158)(2)	e	4.2	1.30
7 - *P. putida* (pXL1289)	d	2.1	0.98
8 - *P. putida* (pXL2169)	d	2.3	0.98

ABBREVIATIONS: a: LB medium; b: LB medium + 100 µg/ml of Amp; c: medium b + addition of 1 mM IPTG to OD_{660nm} = 1; d: LB medium + 50 mg/l of kanamycin; e: M9 medium + 100 mg/l of ampicillin + 50 mg/l of kanamycin; DW: dry weight

COMMON CONDITIONS: 1 to 3: Inoculation in a ratio of 1/100 with a 16-hour-old preculture; culture time 5.75 h; T 37°C
4 to 8: Inoculation in a ratio of 1/100 with a 17-hour-old preculture at 37°C with the addition of tryptophan; culture time in 15 l fermenter: 23 h for *E. coli* and 7.5 h for *P. putida*; T 30°C

2 - SPECIFIC ACTIVITY MEASUREMENTS:

The conditions of the specific activity measurements and the results are collated in Table 5.

TABLE 5: DETERMINATION OF THE ACTIVITIES OF THE CONTROL STRAINS FOR THE RECOMBINANT STRAINS

MICROORGANISM			OPERATING CONDITIONS				Activity U mg of DW
Nature	IPTG	State	Substrate	[DW] (g/l)	Volume (ml)	pH	
1 - *E. coli* TG1	-	W	CVA	15.5	1	5.2	0
	-	W	CVA	15.5	1	7.0	0
	-	W	AdN	15.5	1	5.2	0
	-	W	AdN	15.5	1	7.0	0
2 - *E. coli* TG1 pXL2087	+	W	CVA	1.4	1	4.0	28
	+	W	CVA	1.4	1	5.2	27
	+	W	CVA	1.4	2	7.0	8
	+	S	CVA	1.4	1	5.2	25
	+	S	CVA	1.4	2	7.0	8
	+	W	AdN	0.3	1	4.2	159(a)
	+	W	AdN	1.4	1	4.3	38
	+	W	AdN	1.4	2	6.2	18
	+	W	AdN	1.4	2	7.0	11
	+	S	AdN	1.4	2	6.2	17
	+	S	AdN	1.4	2	7.0	10
3 - *E. coli* TG1 pXL2087	-	W	CVA	1.2	2	4.0	10
	-	W	CVA	1.2	2	5.2	14
	-	W	CVA	1.2	2	7.0	3.4
	-	S	CVA	1.0	1	5.2	13
	-	S	CVA	1.0	1	7.0	3.2
	-	W	AdN	0.3	1	4.2	75(a)
	-	W	AdN	1.2	2	4.3	16
	-	W	AdN	1.2	2	6.2	11
	-	W	AdN	1.2	2	7.0	3.4
	-	S	AdN	1.0	1	6.2	3
	-	S	AdN	1.0	1	7.0	4
4 - *E. coli* TG1 pXL2035	-	W	AdN	0.06	1	7.0	Ua = 0 Ub = not determined
5 - *E. coli* TG1 pXL2168	-	W	AdN	0.24	1	7.0	Ua = 270 Ub = -
			CVA	1.25	1	7.0	Ua = - Ub = 8.3

MICROORGANISM			OPERATING CONDITIONS				Activity U mg of DW
Nature	IPTG	State	Substrate	[DW] (g/l)	Volume (ml)	pH	
6 - *E. coli* TG1 pXL2035, 2158	-	W	AdN	0.06	1	7.0	Ua = 1500 Ub = -
			CVA	0.2	1	7.0	Ua = - Ub = 70
7 - *P. putida* G2081 pDSK519	-	W	AdN	0.3	1	7.0	Ua = 0 Ub = -
8 - *P. putida* G2081 pXL2169	-	W	CVA	0.25	1	7.0	Ua = 130 Ub = -

COMMON CONDITIONS: [substrate] = 50 mM; T = 25°C; buffer 50 mM; kinetics over 90 min for 1 to 3 and over 120 min for 4 to 8

ABBREVIATIONS: W: whole cells; S: sonicated cells; U: 1 µmol of adipate produced/h, except (a) 1 µmol of 5-cyanovalerate produced/h; Ua = µmol of cyanovalerate produced/h/mg of cells DW; Ub = µmol of adipate produced/h/mg of dry cells; AdN: adiponitrile; CVA: 5-cyanovalerate; DW: dry weight

EXAMPLE 7: SYNTHESIS OF AMMONIUM ADIPATE BY THE BATCH HYDROLYSIS OF ADIPONITRILE WITH *E. coli* (pXL2087) IN SUSPENSION

120 µl or 1068 µmol of adiponitrile were added at 25°C and with magnetic stirring, at the reaction times 0, 1, 2, 3, 5, 6 and 7 h, to an initial volume of 5 ml of 50 mM phosphate buffer, pH 7, containing the strain *E. coli* (pXL2087) at an initial concentration of 21 g/l. The reaction was monitored analytically by taking 100 µl samples of the reaction volume every hour. The hydrolysis was found to proceed without a notable loss of kinetics.

The mean activities calculated over 30 min after addition of the adiponitrile are collated in

Table 6 below.

TABLE 6: MEAN ACTIVITIES OF THE *E. coli* (pXL2087) CELLS DURING THE HYDROLYSIS OF ADIPONITRILE

REACTION TIME (h)	SPECIFIC ACTIVITY µmol of adipate h x mg of cells
0.5	16
2.5	15
5.5	11
6.5	11
7.7	15

EXAMPLE 8: SYNTHESIS OF AMMONIUM ADIPATE BY THE HYDROLYSIS OF ADIPONITRILE IN A FIXED BED REACTOR WITH *E. coli* (pXL2087) IMMOBILIZED ON RESIN

The *E. coli* (pXL2087) cells were first fixed by the technique described in US patent 4 732 851.

The resulting biocatalyst was then used in a fixed bed column for the hydrolysis of adiponitrile to ammonium adipate.

1 - FIXING OF *E. coli* (pXL2087) TO RESIN:

The cells were fixed according to the following protocol:

- 1 g (wet weight) of *E. coli* (pXL2087) with a solids content of 22%
- 1 g of POLYCUP polyazetidine
- 1 g of DUOLITE A 171 resin

The gram of cells was suspended in the polyazetidine solution. After homogenization, the resin

was poured into the cell suspension. The whole was stirred with a spatula and then left to dry for 18 h, open to the air, under a hood. 4 ml or 1.3 g of biocatalyst were thus collected.

The activities of the immobilized and free cells were determined at 25°C and pH 7 on a 50 mM solution of adiponitrile. They are respectively 30 and 110 µmol of 2-cyanovalerate/h/mg of cells DW, from which a fixing yield of 26% is deduced.

2 - HYDROLYSIS OF ADIPONITRILE IN A FIXED BED REACTOR:

The half-life is determined in a continuously fed fixed bed reactor under the conditions indicated below:

T 28°C; catalyst 0.5 g or 2 ml or 85 mg of cells (dry weight); [adiponitrile] 50 mM; phosphate buffer 50 mM, pH 7; flow rate 3.7 ± 0.1 ml/h; column: diameter 1 cm, height 3 cm.

The initial activity of the cells was 1.5 µmol of adipate/h/mg of cells (dry weight). 66% of the initial activity is preserved after 32 days or 770 h.

WHAT IS CLAIMED IS:

1. A DNA sequence coding for a polypeptide having a nitrilase activity and capable of hydrolyzing nitriles to carboxylates, which is selected from:
- the DNA sequence coding for a polypeptide having a nitrilase activity, as shown in Fig. 4,
- an analog of this sequence resulting from the degeneracy of the genetic code, and
- a DNA sequence hybridizing with one of these sequences or a fragment thereof and coding for a polypeptide having a nitrilase activity.

2. A recombinant DNA sequence according to claim 1 which contains the following nucleotide sequence:

```
CTCGAGACAA AATTGGGACA GTCGCCCCCT ATCTGCAAAA TGGAACCTCC TTTGCACATC      60
TATAAAATTT TTTGAGGAAG ACAGCAATGA AAAATTATCC TACAGTCAAG GTAGCAGCAG     120
TGCAAGCTGC TCCTGTATTT ATGAATCTAG AGGCAACAGT AGATAAAACT TGTAAGTTAA     180
TAGCAGAAGC AGCATCTATG GGCGCCAAGG TTATCGGCTT CCCAGAAGCA TTTATTCCCG     240
GCTATCCATA TTGGATTTGG ACATCAAATA TGGACTTCAC TGGAATGATG TGGGCCGTCC     300
TTTTCAAGAA TGCGATTGAA ATCCCAAGCA AAGAAGTTCA ACAAATTAGT GATGCTGCAA     360
AAAAGAATGG AGTTTACGTT TGCGTTTCTG TATCAGAGAA AGATAATGCC TCGCTATATT     420
TGACGCAATT GTGGTTTGAC CCGAATGGTA ATTTGATTGG CAAGCACAGG AAATTCAAGC     480
CCACTAGTAG TGAAAGAGCT GTATGGGGAG ATGGGGATGG AAGCATGGCT CCCGTATTTA     540
AAACAGAGTA TGGGAATCTT GGGGGACTCC AGTGCTGGGA ACATGCTCTC CCATTAAACA     600
TTGCGGCGAT GGGCTCATTG AACGAACAGG TACATGTTGC TTCCTGGCCA GCCTTCGTCC     660
CTAAAGGCGC AGTATCATCC AGAGTATCAT CCAGCGTCTG TGCGTCTACT AATGCGATGC     720
ATCAGATCAT TAGTCAGTTT TACGCGATCA GCAATCAGGT ATATGTAATT ATGTCAACCA     780
ATCTCGTTGG CCAAGACATG ATTGACATGA TTGGGAAAGA TGAATTTTCC AAAAACTTTC     840
TACCGCTTGG TTCTGGAAAC ACAGCGATTA TTTCTAACAC CGGTGAGATT TTGGCATCAA     900
TTCCACAAGA CGCGGAGGGA ATTGCTGTTG CAGAGATTGA CCTTAACCAA ATAATTTATG     960
GAAAGTGGTT ACTGGATCCC GCCGGTCATT ACTCTACTCC CGGCTTCTTA AGTTTGACAT    1020
TTGATCAGTC TGAACATGTA CCCGTAAAAA AAATAGGTGA GCAGACAAAC CATTTCATCT    1080
CTTATGAAGA CTTACATGAA GATAAAATGG ATATGCTAAC GATTCCGCCG AGGCGCGTAG    1140
CCACAGCGTG ATCGCCGCCT CTCGGGGCGT TCGGTTGCTG ATAGCCATCG CCTT          1194
```

3. A polypeptide resulting from the expression of a DNA sequence according to one of claims 1 or 2 and possessing a nitrilase activity.

4. A polypeptide according to claim 3 which comprises the following sequence:

```
Met Lys Asn Tyr Pro Thr Val Lys Val Ala Ala Val Gln Ala Ala Pro
 1               5                  10                  15
Val Phe Met Asn Leu Glu Ala Thr Val Asp Lys Thr Cys Lys Leu Ile
            20                  25                  30
Ala Glu Ala Ala Ser Met Gly Ala Lys Val Ile Gly Phe Pro Glu Ala
        35                  40                  45
Phe Ile Pro Gly Tyr Pro Tyr Trp Ile Trp Thr Ser Asn Met Asp Phe
    50                  55                  60
Thr Gly Met Met Trp Ala Val Leu Phe Lys Asn Ala Ile Glu Ile Pro
65                  70                  75                  80
Ser Lys Glu Val Gln Gln Ile Ser Asp Ala Ala Lys Lys Asn Gly Val
                85                  90                  95
Tyr Val Cys Val Ser Val Ser Glu Lys Asp Asn Ala Ser Leu Tyr Leu
            100                 105                 110
Thr Gln Leu Trp Phe Asp Pro Asn Gly Asn Leu Ile Gly Lys His Arg
        115                 120                 125
Lys Phe Lys Pro Thr Ser Ser Glu Arg Ala Val Trp Gly Asp Gly Asp
    130                 135                 140
Gly Ser Met Ala Pro Val Phe Lys Thr Glu Tyr Gly Asn Leu Gly Gly
145                 150                 155                 160
Leu Gln Cys Trp Glu His Ala Leu Pro Leu Asn Ile Ala Ala Met Gly
                165                 170                 175
Ser Leu Asn Glu Gln Val His Val Ala Ser Trp Pro Ala Phe Val Pro
            180                 185                 190
Lys Gly Ala Val Ser Ser Arg Val Ser Ser Ser Val Cys Ala Ser Thr
        195                 200                 205
Asn Ala Met His Gln Ile Ile Ser Gln Phe Tyr Ala Ile Ser Asn Gln
    210                 215                 220
```

Val Tyr Val Ile Met Ser Thr Asn Leu Val Gly Gln Asp Met Ile Asp
225 230 235 240
Met Ile Gly Lys Asp Glu Phe Ser Lys Asn Phe Leu Pro Leu Gly Ser
245 250 255
Gly Asn Thr Ala Ile Ile Ser Asn Thr Gly Glu Ile Leu Ala Ser Ile
260 265 270
Pro Gln Asp Ala Glu Gly Ile Ala Val Ala Glu Ile Asp Leu Asn Gln
275 280 285
Ile Ile Tyr Gly Lys Trp Leu Leu Asp Pro Ala Gly His Tyr Ser Thr
290 295 300
Pro Gly Phe Leu Ser Leu Thr Phe Asp Gln Ser Glu His Val Pro Val
305 310 315 320
Lys Lys Ile Gly Glu Gln Thr Asn His Phe Ile Ser Tyr Glu Asp Leu
325 330 335
His Glu Asp Lys Met Asp Met Leu Thr Ile Pro Pro Arg Arg Val Ala
340 345 350
Thr Ala

5. A microorganism containing the DNA sequence according to claims 1 or 2.

6. A microorganism containing the DNA sequence according to claims 1 or 2 on a plasmid containing a selection means.

7. A microorganism consisting of the strain *E. coli* TG1 containing plasmid pXL2148, said strain having the reference G4207 and being deposited in the Collection Nationale de Cultures de Micro-organismes under no. I-1242.

8. A microorganism containing an expression cassette consisting of the DNA sequence according to claim 1 or 2 under the dependence of signals ensuring the expression of this sequence in the host microorganism.

9. A microorganism according to claim 8 which comprises, upstream from the DNA sequence, a ribosome binding site and a promoter sequence homologous or

heterologous with the polypeptide produced.

10. A microorganism according to claim 9 wherein the promoter can be the tryptophan operon promoter Ptrp of *E. coli*, the lactose operon promoter Plac of *E. coli*, the phage lambda right promoter P_R, the phage lambda left promoter P_L or strong promoters of *Corynebacterium, Comamonas* or *Pseudomonas*.

11. A microorganism according to claim 9 wherein the ribosome binding site can be the one derived from the phage lambda CII gene or those derived from genes of *E. coli, Comamonas, Pseudomonas* or *Corynebacterium*.

12. A microorganism according to claims 8 to 11 wherein the expression cassette is carried by a plasmid containing a selection means.

13. A microorganism according to claim 12 wherein the selection means is a marker conferring antibiotic resistance.

14. A microorganism according to claims 5 to 13 which is selected from the strains of *E. coli, Comamonas, Corynebacterium, Brevibacterium, Rhodococcus* and *Pseudomonas*.

15. A microorganism according to any one of claims 5 to 14

- which contains at least one protein agent for assisting the folding of the polypeptides which the microorganism synthesizes, and in particular the polypeptides according to claim 3 or claim 4, and/or the genes coding for such an agent,
- and wherein this agent is present in a greater amount than that corresponding to the base level of the microorganism in question.

16. A microorganism according to claim 15 wherein the agent is the GroE chaperone of *E. coli* or its homolog of eucaryotic or procaryotic origin.

17. A microorganism according to claim 15 or claim

16 wherein the genes coding for the agent are carried by the chromosome or by an extrachromosomal element (plasmid, phage) and wherein said genes are amplified.

18. A microorganism according to claim 17 wherein the genes coding for the agent are under the dependence of expression systems homologous or heterologous with said microorganism.

19. An enzymatic method of converting nitriles, which consists in bringing the nitriles into contact with a polypeptide having a nitrilase activity, according to either one of claims 3 or 4, or a host microorganism according to any one of claims 5 to 19.

20. A method according to claim 19 wherein the nitrile is a dinitrile of the formula NC-R-CN, in which R is an alkylene group having from 1 to 10 carbon atoms.

21. A method according to one of claims 19 and 20 wherein the nitrile is adiponitrile.

IN THE CANADIAN PATENT AND TRADEMARK OFFICE

PATENT APPLICATION

entitled: Polypeptides possessing a nitrilase activity, DNA sequence coding for said polypeptides, expression cassettes and host microorganisms enabling them to be obtained, and method of converting nitriles to carboxylates by means of said polypeptides

in the names of: Dominique PETRE
Edith CERBELAUD
Sophie LEVY-SCHIL
Joël CROUZET

Assignee: RHONE POULENC CHIMIE

ABSTRACT OF THE DISCLOSURE

The present invention relates to novel polypeptides having a nitrilase activity and to the genetic tools for producing them, namely:

- the DNA sequence coding for a polypeptide having a nitrilase activity and capable of hydrolyzing nitriles to carboxylates,
- an analog of this sequence resulting from the degeneracy of the genetic code,
- a DNA sequence hybridizing with one of these sequences or a fragment thereof and coding for a polypeptide having a nitrilase activity, and
- expression cassettes and microorganisms enabling them to be obtained.

Application: enzymatic conversion of nitriles to carboxylates.

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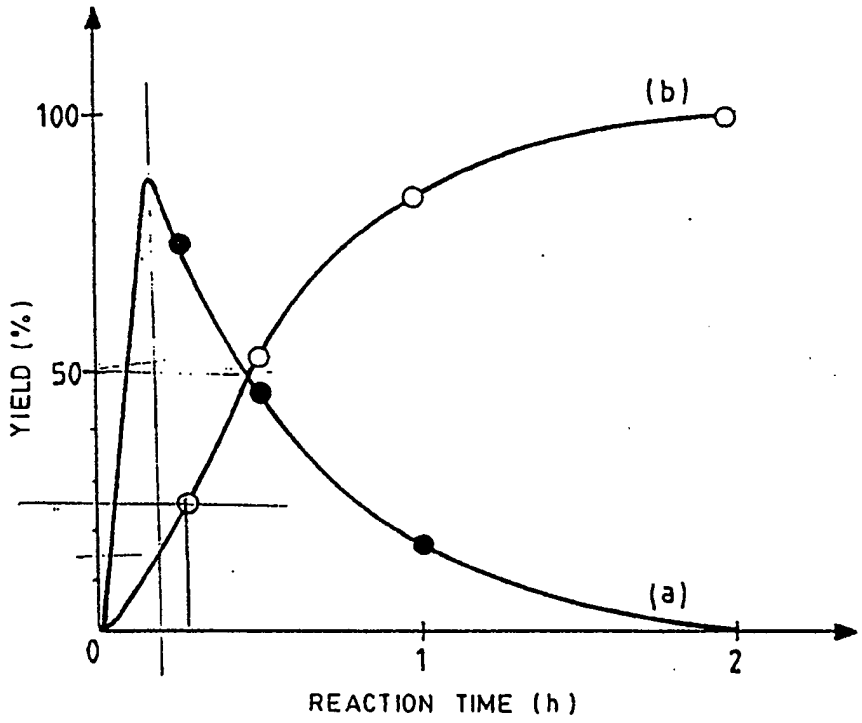


FIG.1

2103616

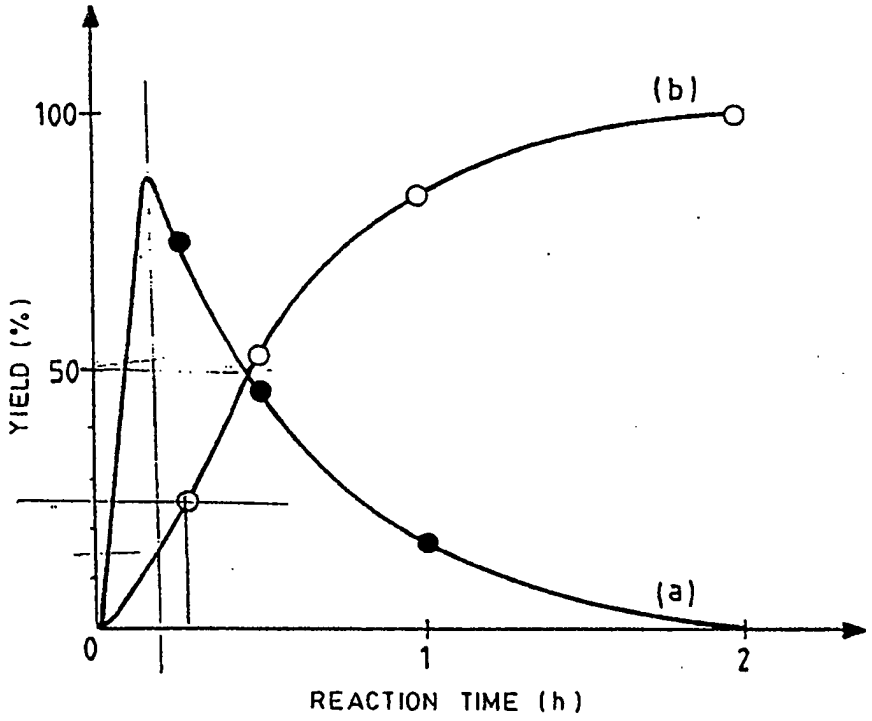


FIG.1

FIG. 2a

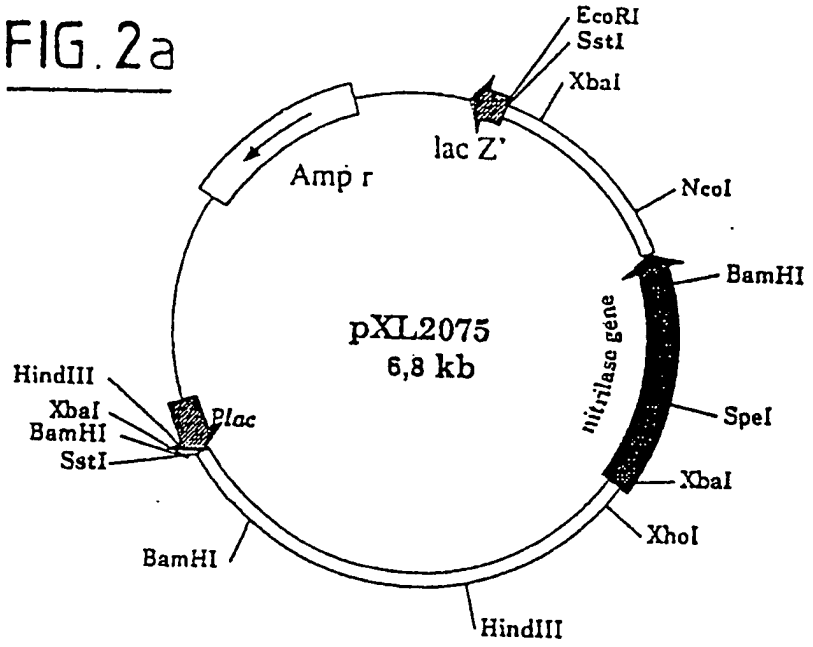
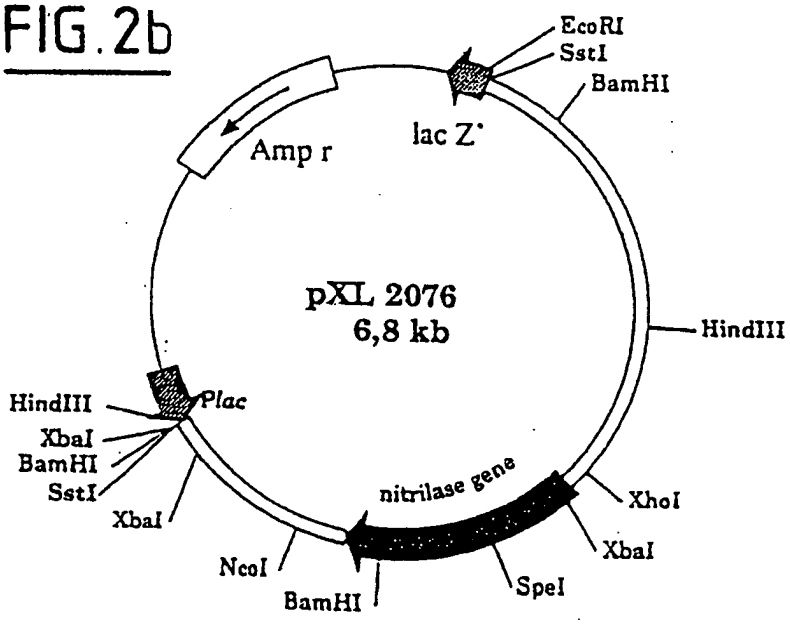

EcoRI
SstI
XbaI
lac Z'
Amp r
NcoI
BamHI
pXL2075
6,8 kb
nitrilase gène
SpeI
XbaI
XhoI
HindIII
XbaI
BamHI
SstI
Plac
BamHI
HindIII

FIG. 2b


EcoRI
SstI
BamHI
Amp r
lac Z'
pXL 2076
6,8 kb
HindIII
HindIII
Plac
XbaI
BamHI
SstI
XbaI
nitrilase gene
XhoI
XbaI
NcoI
BamHI
SpeI

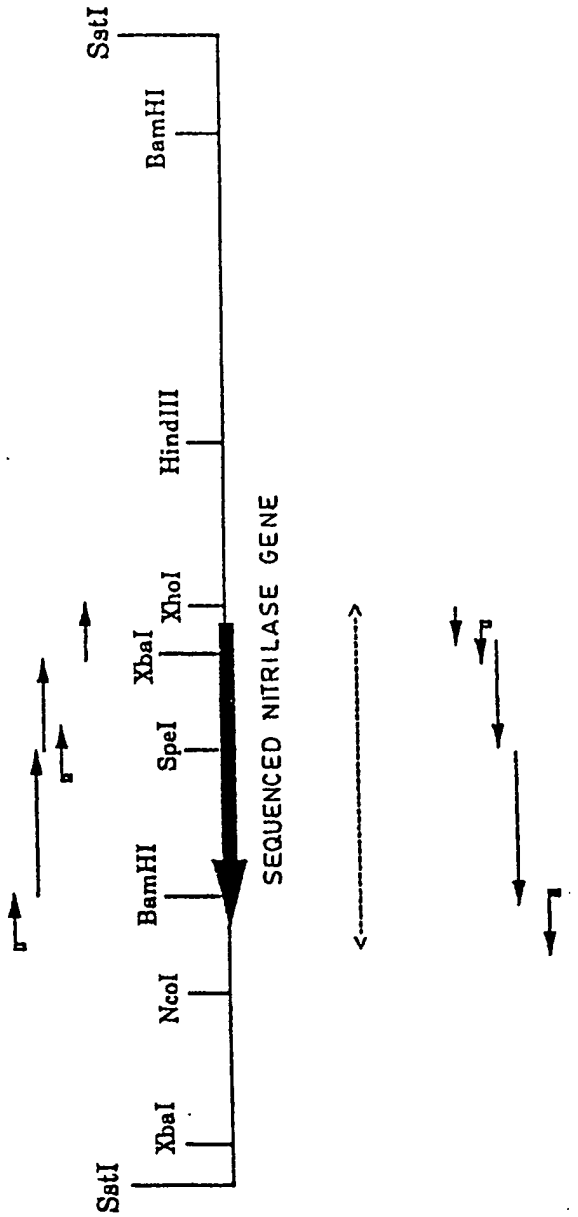

SstI
BamHI
HindIII
XhoI
XbaI
SpeI
BamHI
NcoI
XbaI
SstI
SEQUENCED NITRILASE GENE
200bp
SPECIFIC OLIGONUCLEOTIDE
UNIVERSAL PRIMER

FIG. 3

```
ctcgagacaaaattgggacagtcgcccctatctgcaaaatggaacctcctttgcacatctataaaatttttt   72

tgaggaagacagca ATG AAA AAT TAT CCT ACA GTC AAG GTA GCA GCA GTG CAA GCT   128
               Met Lys Asn Tyr Pro Thr Val Lys Val Ala Ala Val Gln Ala    14

GCT CCT GTA TTT ATG AAT CTA GAG GCA ACA GTA GAT AAA ACT TGT AAG TTA ATA   182
Ala Pro Val Phe Met Asn Leu Glu Ala Thr Val Asp Lys Thr Cys Lys Leu Ile    32

GCA GAA GCA GCA TCT ATG GGC GCC AAG GTT ATC GGC TTC CCA GAA GCA TTT ATT   236
Ala Glu Ala Ala Ser Met Gly Ala Lys Val Ile Gly Phe Pro Glu Ala Phe Ile    50

CCC GGC TAT CCA TAT TGG ATT TGG ACA TCA AAT ATG GAC TTC ACT GGA ATG ATG   290
Pro Gly Tyr Pro Tyr Trp Ile Trp Thr Ser Asn Met Asp Phe Thr Gly Met Met    68

TGG GCC GTC CTT TTC AAG AAT GCG ATT GAA ATC CCA AGC AAA GAA GTT CAA CAA   344
Trp Ala Val Leu Phe Lys Asn Ala Ile Glu Ile Pro Ser Lys Glu Val Gln Gln    86

ATT AGT GAT GCT GCA AAA AAG AAT GGA GTT TAC GTT TGC GTT TCT GTA TCA GAG   398
Ile Ser Asp Ala Ala Lys Lys Asn Gly Val Tyr Val Cys Val Ser Val Ser Glu   104

AAA GAT AAT GCC TCG CTA TAT TTG ACG CAA TTG TGG TTT GAC CCG AAT GGT AAT   452
Lys Asp Asn Ala Ser Leu Tyr Leu Thr Gln Leu Trp Phe Asp Pro Asn Gly Asn   122

TTG ATT GGC AAG CAC AGG AAA TTC AAG CCC ACT AGT AGT GAA AGA GCT GTA TGG   506
Leu Ile Gly Lys His Arg Lys Phe Lys Pro Thr Ser Ser Glu Arg Ala Val Trp   140

GGA GAT GGG GAT GGA AGC ATG GCT CCC GTA TTT AAA ACA GAG TAT GGG AAT CTT   560
Gly Asp Gly Asp Gly Ser Met Ala Pro Val Phe Lys Thr Glu Tyr Gly Asn Leu   158

GGG GGA CTC CAG TGC TGG GAA CAT GCT CTC CCA TTA AAC ATT GCG GCG ATG GGC   614
Gly Gly Leu Gln Cys Trp Glu His Ala Leu Pro Leu Asn Ile Ala Ala Met Gly   176
```

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FIG.4

(SEE OVER)

```
TCA TTG AAC GAA CAG GTA CAT GTT GCT TCC TGG CCA GCC TTC GTC CCT AAA GGC   668
Ser Leu Asn Glu Gln Val His Val Ala Ser Trp Pro Ala Phe Val Pro Lys Gly   194

GCA GTA TCA TCC AGA GTA TCA TCC AGC GTC TGT GCG TCT ACT AAT GCG ATG CAT   722
Ala Val Ser Ser Arg Val Ser Ser Ser Val Cys Ala Ser Thr Asn Ala Met His   212

CAG ATC ATT AGT CAG TTT TAC GCG ATC AGC AAT CAG GTA TAT GTA ATT ATG TCA   776
Gln Ile Ile Ser Gln Phe Tyr Ala Ile Ser Asn Gln Val Tyr Val Ile Met Ser   230

ACC AAT CTC GTT GGC CAA GAC ATG ATT GAC ATG ATT GGG AAA GAT GAA TTT TCC   830
Thr Asn Leu Val Gly Gln Asp Met Ile Asp Met Ile Gly Lys Asp Glu Phe Ser   248

AAA AAC TTT CTA CCG CTT GGT TCT GGA AAC ACA GCG ATT ATT TCT AAC ACC GGT   884
Lys Asn Phe Leu Pro Leu Gly Ser Gly Asn Thr Ala Ile Ile Ser Asn Thr Gly   266

GAG ATT TTG GCA TCA ATT CCA CAA GAC GCG GAG GGA ATT GCT GTT GCA GAG ATT   938
Glu Ile Leu Ala Ser Ile Pro Gln Asp Ala Glu Gly Ile Ala Val Ala Glu Ile   284

GAC CTT AAC CAA ATA ATT TAT GGA AAG TGG TTA CTG GAT CCC GCC GGT CAT TAC   992
Asp Leu Asn Gln Ile Ile Tyr Gly Lys Trp Leu Leu Asp Pro Ala Gly His Tyr   302

TCT ACT CCC GGC TTC TTA AGT TTG ACA TTT GAT CAG TCT GAA CAT GTA CCC GTA  1046
Ser Thr Pro Gly Phe Leu Ser Leu Thr Phe Asp Gln Ser Glu His Val Pro Val   320

AAA AAA ATA GGT GAG CAG ACA AAC CAT TTC ATC TCT TAT GAA GAC TTA CAT GAA  1100
Lys Lys Ile Gly Glu Gln Thr Asn His Phe Ile Ser Tyr Glu Asp Leu His Glu   338

GAT AAA ATG GAT ATG CTA ACG ATT CCG CCG AGG CGC GTA GCC ACA GCG TGA tcgc  1155
Asp Lys Met Asp Met Leu Thr Ile Pro Pro Arg Arg Val Ala Thr Ala           354

cgcctctcggggcgttcggttgctgatagccatcgcctt                                 1194
```

FIG.4

(CONTINUATION)

2103615

FIG.5

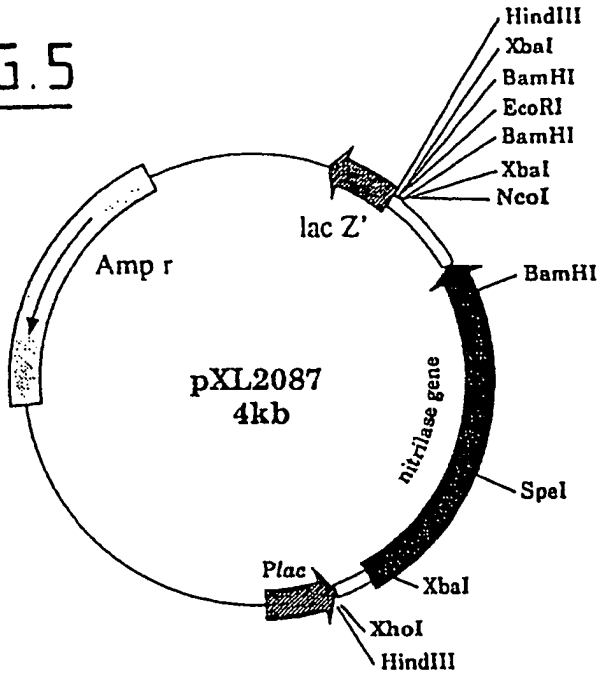
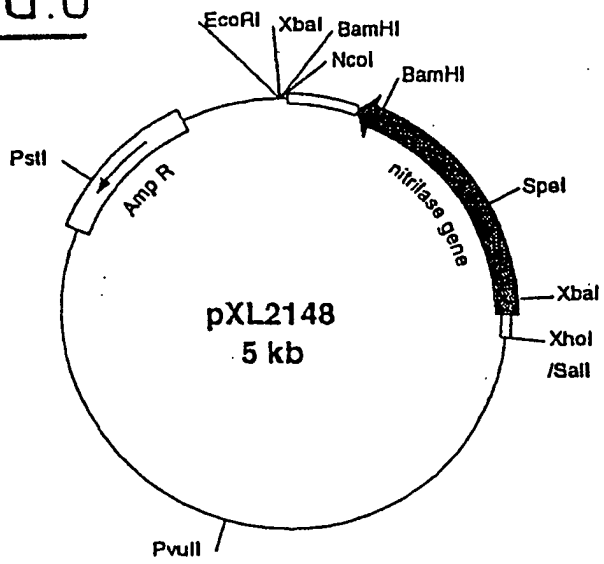

HindIII
XbaI
BamHI
EcoRI
BamHI
XbaI
NcoI
lac Z'
Amp r
BamHI
pXL2087
4kb
nitrilase gene
SpeI
Plac
XbaI
XhoI
HindIII

FIG.6


EcoRI
XbaI
BamHI
NcoI
BamHI
PstI
Amp R
nitrilase gene
SpeI
XbaI
pXL2148
5 kb
XhoI
/SalI
PvuII

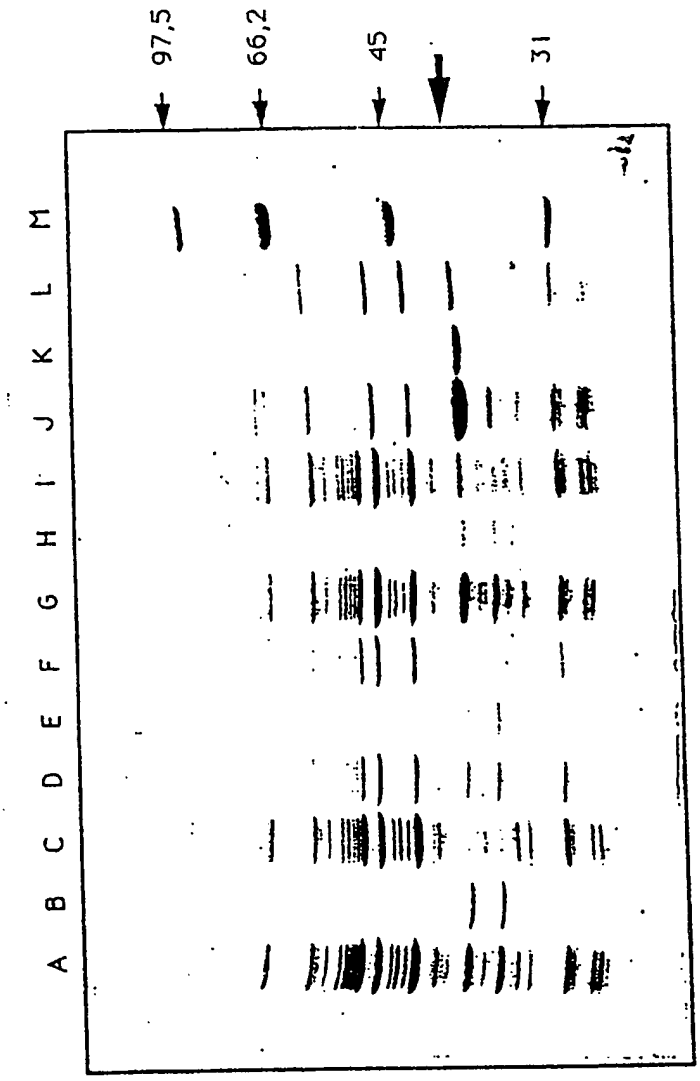


FIG.7

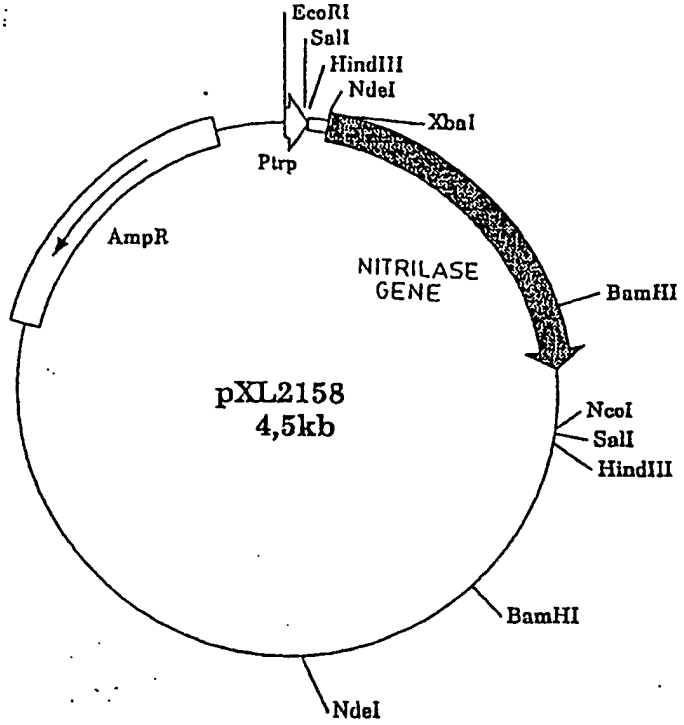

EcoRI
SalI
HindIII
NdeI
XbaI
Ptrp
AmpR
NITRILASE
GENE
BamHI
pXL2158
4,5kb
NcoI
SalI
HindIII
BamHI
NdeI

FIG. 8

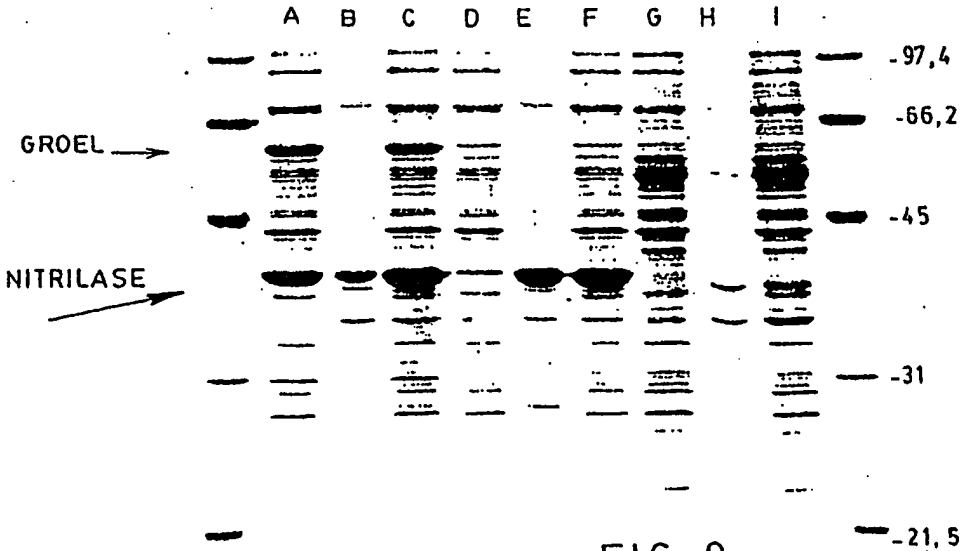

A
B
C
D
E
F
G
H
I
GROEL
NITRILASE
-97,4
-66,2
-45
-31
-21,5

FIG. 9

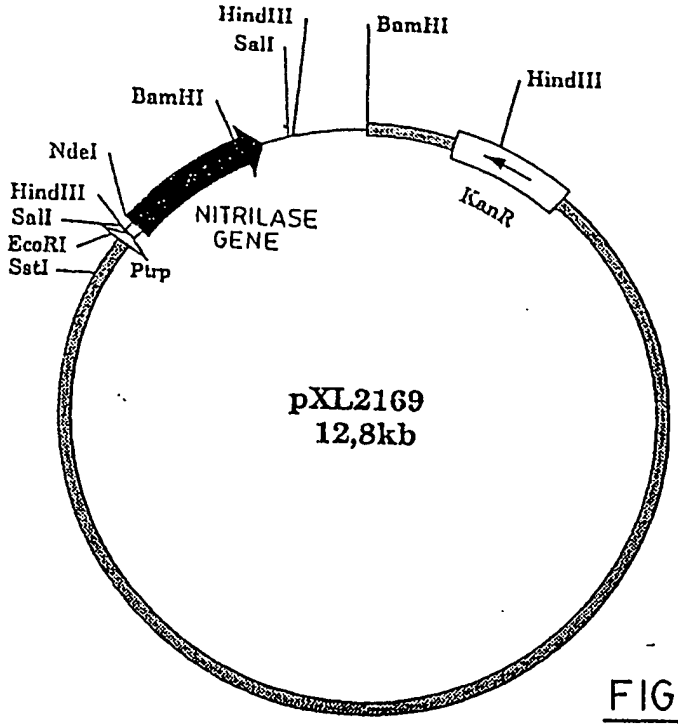

HindIII
SalI
BamHI
BamHI
HindIII
NdeI
HindIII
SalI
EcoRI
SstI
Ptrp
NITRILASE
GENE
KanR
pXL2169
12,8kb

FIG. 10


A
B
C
D
NITRILASE
-97,4
-66,2
-45
-31
-21,5

FIG. 11